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RESEARCH

Modulating the growth, immune/antioxidant traits, immune-related genes, and resistance to *Aeromonas veronii* infection in *Oreochromis niloticus* reared under high stocking densities by dietary *Lavandula angustifolia* essential oil

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Abstract

In this study, we investigated the effects of the dietary addition of lavender essential oil (LEO) on mitigating the adverse effects of high stocking densities (HSD) in the Nile tilapia, *Oreochromis niloticus.* We used 216 fingerlings and randomly distributed them according to a 2×3 factorial design, comprising two stocking densities: a low stocking density (LSD, 2.92 kg/m^3) and a high stocking density (5.85 kg/m^3) and three levels of LEO, 0, 0.5%, and 1%, for 70 days. We evaluated their growth, digestive enzyme activity, blood biochemical parameters, immune/antioxidant indices, and intestinal morphology. All fish were intraperitoneally inoculated with *Aeromonas veronii* for a bacterial challenge test at the end of the feeding trial and monitored for 14 days. After 14 days, head kidney samples were collected from all fish to assess the level of expression of immune-related and apoptosis-related genes. Additionally, tissue samples were collected from the liver, kidney, and intestine to investigate the immunohistochemistry and histopathological alterations. The results showed that the final body weight and weight gain were lower and the feed conversion ratio was higher in fish cultured under HSD compared to those cultured under LSD. The fish cultured under LSD and HSD had higher final body weight when administered dietary LEO compared to the fish that were fed the non-supplemented diet. Adding 1% LEO increased the intestinal villus height and muscular coat thickness (MT). The MT was lower in fish cultured under HSD compared to those cultured under LSD. The amylase and lipase activities were lower in the fish cultured under HSD than in those cultured under LSD. However, LEO addition increased the lipase activity in fish cultured under LSD and HSD. Fish cultured under HSD showed lower immune/antioxidant indices (lysozymes, nitric oxide, complement 3, catalase, superoxide dismutase, and glutathione-S transferase) than those cultured under LSD. However, LEO addition significantly increased the immune response and antioxidant activity in fish cultured under LSD and HSD ($P < 0.01$). The level of expression of the *IL-1β*, *IL-6*, *IL-8, NF-kB*, and *TLR-4 B* genes was upregulated in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups. The expression of *TGF-β* and

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IL-10 was upregulated in fish that received 1% and 0.5% LEO, irrespective of whether they were cultured under LSD or HSD. The level of expression of apoptosis-related genes (*caspase 3* and *BAX*) was upregulated in the HSD + LEO0 group and modified by the addition of LEO in their diet. The immunoexpression of *NF-kB* and *IL-1β* after bacterial challenge increased in the tissues of fish cultured under LSD and HSD after LEO was administered. These results indicated that the dietary addition of 1% LEO mitigated the harmful effects of HSD on the growth, immunity, antioxidant activity, and disease resistance of the Nile tilapia.

Keywords *Oreochromis niloticus* · Phytobiotics · Stress · Immunity · *Aeromonas veronii*

Introduction

In fish farms, fish are stressed by handling, grouping, shipping, crowding, etc. (Ashley 2007). The health of fish can deteriorate under stress in different ways. Stressful conditions increase the levels of stress-related biomarkers, which increases energy expenses (described by hyperglycemia) and inhibits growth (Andrade et al. 2015; Yousefi et al. 2016). Additionally, the release of high levels of stress-related hormones is associated with immunosuppression due to the inhibition of inflammatory reactions (Mirghaed et al. 2018; Ghelichpour et al. 2019). Stressors also disrupt the antioxidant mechanism, leading to oxidative stress (Mirghaed et al. 2018). Hence, stress factors facilitate the rapid spread of infectious diseases in various aquaculture resources, due to the disruption of their immune response; consequently, fish under stress become more vulnerable to infection (Mahboub et al. 2022). Fish diseases cause enormous annual losses to the aquaculture industry worldwide, and antibiotic treatment not only is very expensive but also has many harmful effects, such as the emergence of antibiotic-resistant bacteria and residual deposition in the edible tissues of fish (Ahmed et al. 2020).

Stocking density is an important factor that affects net fish production and the profitable outcomes of tilapia culture (Naderi et al., 2019). Several researchers have experimentally demonstrated the effect of rearing density on various health attributes of fish. They found that fish growth, behavior, immune status, and survival were the critical factors affected by the rearing density (Ashley 2007; El-Sayed 2002; Huntingford et al. 2006). Although rearing fish at high stocking densities has some limitations, including relatively low growth rates and higher prevalence of diseases, most pisciculturists around the world use this approach. However, whether these problems arise due to higher culture densities or are associated with water quality deterioration is unclear (Ellis et al. 2002; Shourbela et al. 2017). *Oreochromis niloticus* specimens, when cultured at low densities (300 fish/m^3), showed a significantly higher growth rate than those cultured at high densities (600 fish/m^3) (Yakubu et al. 2012). Additionally, reductions in growth, ingestive behavior, and immune status were observed at higher rearing densities (Lupatsch et al. 2010; Ellis et al. 2002).

To counteract the side effects of higher rearing densities, functional feed additives are good antibiotic candidates as they improve the health of fish. An efficient additive can boost the health of animals by increasing feed digestibility and biodegradation of nutrients (Grisdale-Helland et al. 2008), and also by activating their immune system (Torrecillas et al. 2014), mitigating stress response (Torrecillas et al. 2012), and enhancing the integrity of tissues (Zhou et al. 2010). Hence, fish farmers aim to reduce stress, increase the health and immunity of fish, prevent diseases, and minimize the adverse effects of

antibiotic treatment. Phytotherapy has attracted the attention of the aquaculture industry. Medicinal plants are valuable feed additives that are naturally produced and eco-friendly (Amer et al. 2018, 2022b; El-Araby et al. 2022; Ahmed et al. 2023; Al-Khalaifah et al. 2020). Researchers have investigated the effectiveness of different forms of plant products, including whole plants, extracts, essential oils, and active ingredients, in different types of fish for their health benefits. Additionally, plant feed additives were found to improve the immune system of fish (Amer et al. 2018) and prolong the survival of fish during disease outbreaks (El-Araby et al. 2022). Several studies have reported that plant feed additive compounds can prompt anti-stress properties in broiler chickens (Hosseini et al. 2016) and fish (Mohiseni et al. 2017).

Lavender (*Lavandula angustifolia*) is a medicinal plant known for its antioxidant (Gülçin et al. 2004; Amer et al. 2022a), anti-inflammatory (Cardia et al. 2018), and hepatoprotective (Selmi et al. 2015) effects. Lavender essential oil has aromatherapeutic, antifungal, and antibacterial properties, mostly due to the presence of compounds such as lavandulol, eucalyptol, linalyl acetate, linalool, or geraniol (Białoń et al. 2019). It also contains polyphenols, such as flavonoids, which have several chemical and biological activities with radical scavenging properties (Rabiei et al. 2014).

In this study, we investigated the effects of dietary LEO on mitigating the adverse effects that occur when Nile tilapia are reared under high stocking densities; we specifically focused on the growth metrics, intestinal histo-morphology, activity of digestive enzymes, blood biochemical parameters, immune indices, antioxidant activity, disease resistance, and the expression of immune-related and apoptosis-related genes.

Materials and methods

Lavender essential oil

Lavender essential oil (LEO) was purchased from Organic Egypt, Cairo, Egypt. We determined the bioactive compounds in LEO in another study using the GC-MS technique (Amer et al. 2022a); the corresponding chromatogram is shown in Fig. 1. The main bioactive compounds identified were α-pinene (32.24%), acetic acid linalool ester (32.24%),

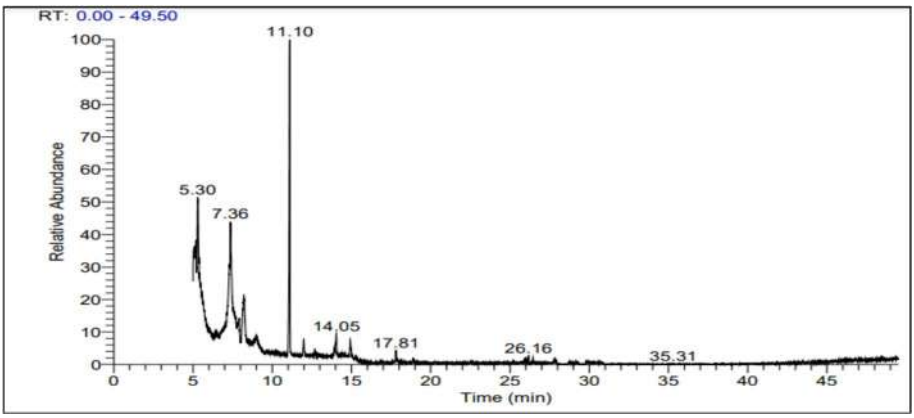

Fig. 1 Chromatographic characteristics by GC-mass techniques

cis-α-terpineol (16.06%), α-linalool (16.06%), eucalyptol (11.74%), phellandral (10.91%), acetic acid (2.69%), geranyl vinyl ether (2.25%), and nerolidyl acetate (2.25%).

The high-performance liquid chromatography (HPLC) analysis of LEO

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using Zorbax Eclipse Plus C8 column (4.6 mm × 250 mm i.d., 5 µm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate 0.9 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–1 min (82% A); 1–11 min (75% A); 11–18 min (60% A); 18–22 min (82% A) ; 22–24 min (82% A). The multi-wavelength detector was monitored at 280 nm. The injection volume was 5 µl for each of the sample solutions. The column temperature was maintained at 40 °C.

Nile tilapia fingerlings

Mono-sex fingerlings of Nile tilapia ($n=216$, mean initial weight: 23.39 ± 0.19 g) were obtained from El-Tal El-Kebir Fish Farm, Ismailia Governorate, Egypt. These fish did not have a history of diseases or clinical anomalies. Their health was examined as per Canadian Council on animal care guidelines CCoA (2005). The fish were randomly distributed into 18 static water glass aquaria (80 cm× 30 cm ×40 cm) containing dechlorinated tap water. We changed 25% of the water in the aquarium daily by siphoning off the organic matter debris and changed it completely twice a week. Water quality parameters were assessed following the method described by APHA (1992). Several parameters were kept constant during the experiment, including temperature (22.4 ± 1.01 °C), dissolved oxygen (7.12 ± 0.4 mg/L), ammonia-N (0.034 ± 0.01 mg/L), nitrite (0.13 ± 0.01 mg/L), and pH (6.8 ± 0.1). All fish were maintained under a 12-h/12-h light/dark photoperiod. To keep all aquariums well-aerated, we used an air bubble stone diffuser connected to a plastic hose and an air blower. The dissolved oxygen level was measured using an electronic oxygen meter (Mettler Toledo, UK). The water temperature was measured using a digital thermometer every 30 min.

Experimental protocol and feeding regimen

All fish were acclimated for 2 weeks before the experiment. While acclimating, they were fed the conventional base diet. Then, they were randomly assigned to a group based on a 2×3 factorial design for 70 days; all trials were conducted in triplicate. The groups consisted of two stocking densities—a low stocking density (LSD, 2.92 kg/m^3) and a high stocking density (5.85 kg/m^3)—and three experimental diets with three inclusion levels of LEO, which were as follows: (1) basal diet with no LEO, (2) basal diet + 0.5% LEO, and (3) basal diet + 1% LEO. The basal diet was formulated according to the recommendations of the National Research Council (NRC 2011) (Table 1). Lavender essential oil (0, 5, and 10 g kg^{-1}) was mixed with the ingredients of the basal diet as a feed additive. Next, water was added to produce a doughy, moist mass. The dough mixture was turned into sinking pellets (2 mm in diameter) using a meat mincer. Finally, the pellets were air-dried at room temperature under regular rotation for 48 h for even drying. After drying, the pellets were kept in sterile, clean plastic bags in the refrigerator at 4 °C until further use. Proximate chemical analysis of the basal diet was

Table 1 Feed formulation and proximate composition (% on a dry weight basis)

Ingredients	Percent
Fish meal 70.7% CP	20
Soybean meal 49% CP	25
Yellow corn	26
Wheat bran	5
Wheat flour	10
Corn gluten 67% CP	5
Fish oil	3
Soy oil	3
Premix [1]	3
Chemical composition (%)	
Crude protein	34.37
Crude fiber	3.47
Fat	10.02
NFE [2]	45.56
Ash	6.56
Lysine	2.03
Methionine	0.72
GE MJ/kg [3]	20.56

CP, crude protein

[1]Composition/kg: Vit. A 580,000 IU; Vit. D3 8600 IU; Vit. K3 142 mg; Vit. E 720 mg; Vit. C 30,000 mg; Vit. B1 58 mg; Vit. B2 34 mg; Vit. B6 34 mg; Vit. B12 58 mg; folic acid 86 mg; biotin 50 mg; pantothenic acid 8 mg; Zn methionine 3000 mg; Mn sulfate 65 mg; copper sulfate 3400 mg; iron sulfate 2000 mg; sodium selenite 25 mg; cobalt sulfate 572 mg; calcium iodide 25 mg; calcium carbonate as carrier up to 1 kg. [2]Nitrogen free extract (NFE), determined by difference = 100 − (protein % + fat % + crude fiber % + ash %). [3]Gross energy (GE) was calculated as 23.6 kJ/g protein, 39.5 kJ/g lipid, and 17.0 kJ/g NFE (NRC 2011)

conducted according to the method described by the Association of Official Analytical Chemists (AOAC) (2000). The fish were fed manually until satiety thrice a day (at 9:00 a.m., noon, and 3:00 p.m.) throughout the experimental duration (70 days). The research program animal care and use committee at Zagazig University in Egypt approved the experimental protocol (ZU-IACUC/2/F/299/2023). All relevant general animal care and use standards were followed while conducting the experiments and handling the animals.

Growth performance parameters

Fish weight and feed intake were determined at the start and end of the trial. Total weight gain (TWG) and feed conversion ratio (FCR) were also calculated.

$$\mathrm{TWG/fish}(g) = \frac{\mathrm{FBW(g)/tank} - \mathrm{IBW(g)/the\ same\ tank}}{N}$$

Here, TWG indicates the total weight gain, determined at the end of the experiment, FBW indicates the final body weight of the fish in each tank determined at the end of

the experiment, IBW indicates the initial body weight of fish in the same tank recorded at the start of the experiment, and N indicates the number of fish in the tank.

$$\text{Total FI/fish}(g) = \frac{\text{Total amount of feed intake (g)/tank}}{N}$$
$$\text{FCR} = \text{Total FI (g)/ TWG (g)}$$
$$\text{SGR (\%/day)} = 100 \times \frac{\ln \text{FBW} - \ln \text{IBW}}{\text{time in days}}$$

Here, ln indicates the natural logarithm.

The protein efficiency ratio (PER) was determined using the equation provided in the study by Stuart and Hung (1989).

$$\text{PER} = \text{TWG (g)/ protein intake (g)}$$

Blood and tissue sampling

At the end of the feeding period, three fish per replicate (i.e., nine fish per group) were anesthetized with 100 mg/L of benzocaine solution (Neiffer and Stamper 2009). Blood samples were collected by puncturing the caudal vessels using a 1-mL syringe. The blood sample was collected without any anticoagulant and centrifuged at 3000 rpm for 15 min to obtain the serum. The serum samples collected were used to assess the innate immunity, antioxidant status, and serum biochemical parameters. Liver and intestinal samples ($n = 9$ each) were collected and frozen at − 20 °C to determine the activities of antioxidant and digestive enzymes, respectively.

Intestinal digestive enzymes

The intestines of three fish from each group were carefully extracted and weighed. The intestines were homogenized in a tissue homogenizer with 10 volumes (v/w) of cold saline and placed in an ice bath. Next, the extract was centrifuged for 10 min at 1750×g. The amylase activity was measured in the extract following the methods described by Worthington (1993). This approach used an iodine solution to expose the non-hydrolyzed starch, and the activity was reported as "specific activity" (units per mg of protein). Meanwhile, the lipase activity was assessed via a spectrofluorometer, according to the procedures previously mentioned by Izquierdo and Henderson (1998). Neutral lipase assays was conducted using acyl esters of 4-methylumbelliferone and 1-acyl-2-[6 (7 nitro-1,3 benzoxadiazol-4-yl) amino]caproyl labelled phosphatidylcholine compounds (NBD-PC), respectively, as substrates. This was done to investigate the potential of fluorescence-based assays in the study of fish lipid digestion. In contrast to the butyrate or oleate esters, 4-methylumbelliferyl hepatanoate (4-MUH) hydrolyzed more quickly. Additionally, the hexanoic (C6) ester of NBD-PC worked better as a substrate for the phospholipase assay than the dodecanoic (C12) ester. Using 50 mm of potassium phosphate buffer pH 7.8 instead of 0.01 m citrate/sodium phosphate buffer pH 7.2 resulted in about 10% more neutral lipase activity. The neutral lipase activity in both experiments was detectable at a very sensitive level.

Serum biochemical assay and protein profile analysis

The level of growth hormone (GH) was determined using a fish growth hormone ELISA kit (MyBioSource Co., Cat. No. MBS044656), following the manufacturer's instructions. Serum glucose levels were estimated using Spectrum Bioscience kits (Egyptian Company for Biotechnology, Cairo, Egypt). Total serum protein levels and the albumin content were assessed following the method described in another study (Doumas et al. 1981). Total globulins were estimated by subtracting the albumin content from the total protein content (Doumas et al. 1972).

Assessment of hepatorenal injury markers

The levels of hepatic alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured using Spinreact kits (Esteve De Bas, Girona, Spain) following the method described by Burtis and Ashwood (1994). The renal creatinine and urea levels were estimated using former kits, following the methods reported by Fossati et al. (Henry 1974) and Patton and Crouch (1977), respectively.

Estimation of immune indices

Lysozyme activity in serum samples was assessed following the protocols reported by Ellis (1990). In a nutshell, 0.75 mg/mL *Micrococcus lysodeikticus* (Sigma) was dissolved in 0.1 M sodium phosphate buffer (pH = 5.9) to create the lysozyme standard suspension. In total, 175 µL of the suspension was then added to a microtiter plate and incubated for 5 min at 30 °C. A 25-µL serum solution was then applied to the microtiter plate. The quantity of serum lysozyme (µg/mL) was determined using the lyophilized hen egg white lysozyme standard curve (Sigma). Serum nitric oxide content was measured according to Montgomery and Dymock (1961). This approach is based on the production of a bright red color as a result of the reaction between nitric oxide (NO), sulfanilamide, and 1-naphthyl ethylenediamine. In addition, complement 3 (C3, catalog no. MBS281020) was evaluated using kits from MyBioSource Co. (San Diego, CA, USA) based on the instructions provided by the manufacturer.

Liver antioxidant indicators

Fresh liver samples ($n = 9$) were used to estimate the hepatic content of antioxidant indicators. The activity of catalase (CAT) was measured following the methods previously described by Aebi (1984) through monitoring the decline in the absorbance of H_2O_2 at 240 nm. The activity of superoxide dismutase (SOD) was evaluated according to the earlier protocol of McCord and Fridovich (1969). The ability of SOD to prevent pyrogallol from auto-oxidizing was estimated to determine the enzyme's activity. The quantity of SOD enzyme that caused a 50% suppression of the pyrogallol auto-oxidation seen in the blank was the unit of SOD enzyme activity. Glutathione S transferase (GST) was evaluated according to the earlier protocol of Habig et al. (1974), where the tissue sample was preincubated for 5 min at 37 °C following the addition of glutathione (0.5 mM) and sodium phosphate buffer (0.1 M), pH 7.3. Subsequently, the incubation mixture was supplemented

with 0.5 mM of 1-chloro-2,4-dinitrobenzene (CDNB). The mixture was then centrifuged after the addition of 33% trichloroacetic acid solution. At 340 nm, the CDNB conjugate in the supernatant was detected. The enzyme's activity was measured as the amount needed to increase the synthesis of 1 µmol of CDNB conjugate/mg protein/min. The hepatic lipid peroxidation marker, malondialdehyde (MDA), was measured in different groups by colorimetric assay, using thiobarbituric acid (TBA) as a reagent, following the protocol of Sochor et al. (2012). This technique depends on monitoring the absorbance of the MDA/TBA adduct, where the samples were mixed with 10% trichloroacetic acid containing 0.25% TBA. The former mixture was heated for 30 min at 95 °C and then centrifuged at 10,000 rpm after cooling. At 532 nm, the supernatant's absorbance was calculated. Utilizing an extinction value of 155 mM^{-1} cm^{-1}, the MDA content was computed.

Disease challenge test

A pathogenic strain of *A. veronii* was recovered from infected Nile tilapia at the Microbiology Unit, Department of Aquatic Animal Medicine, Faculty of Veterinary Medicine, Zagazig University. The isolate was identified using conventional biochemical techniques and 16 S rRNA gene sequencing (Reda et al. 2022). The lethal dose (LD_{50}) of *A. veronii* was found to be 2.4×10^7 CFU/mL. At the end of the 70-day feeding trial, five fish per replicate (15 fish/group) were intraperitoneally inoculated with a sub-lethal dose of *A. veronii* (0.2 mL of cell suspension, 1×10^7 CFU/mL) using McFarland standard tubes. The challenged fish were monitored daily for 2 weeks, and behavioral disorders, clinical signs, and mortality were recorded.

Analysis of immune-related and apoptosis-related gene expression in head kidney tissue

A quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed to assess the changes in the transcriptomic profile of the immune-related and apoptosis-related genes and inflammatory cytokines in the head kidney samples collected from the fish in all groups (nine samples/group) after they were infected with *A. veronii*. Under a septic condition, head kidney samples were carefully collected and frozen in liquid nitrogen to inhibit RNAase activity and preserve RNA integrity. The tissues were then preserved at −80 °C until the total RNA extraction was performed. Trizol (Invitrogen; Thermo Fisher Scientific, Inc.) was used for RNA extraction following the manufacturer's protocol. In total, 30 mg of the head kidney tissue was collected in 1 mL of Trizol. The head kidney tissue was then minced and homogenized with a tissue raptor, and after that, the total RNA was extracted according to manufacturer instructions. Real-time RT-PCR was performed using an Mx3005 P Real-Time PCR System (Agilent Stratagene, USA) and TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX), following the manufacturer's instructions, for gene expression analysis. The PCR cycling conditions were as follows: primary denaturation at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, and extension at 72 °C for 60 s. The melting curve was examined at 65–95 °C in increments of 0.1 °C/s to assess the specificity of the qPCR products. All samples were analyzed in duplicate. Non-template controls

Table 2 The list of primers used for the real-time quantitative PCR amplification in this study

Gene	Primer sequence	Tm	Accession no.	Size	Efficiency
β-Actin	CCACCCAAAGTTCAGCCATG ACGATGGAGGGGAAGACAG	59.4 58.4	XM_003443127.5	121	98.5
IL-1β	CTCATGTCTGTCCGCTACCC TGAAGCTTCTGTAGCGTGGG	59.9 60.04	KF747686.1	103	96
TGF-β	CCAGAGCAGAGCTACGGATG CCAGGTCTGCAGAGGTTCAG	59.97 60.04	NM_001311325.1	173	97.5
IL-6	ACAGAGGAGGCGGAGATG GCAGTGCTTCGGGATAGAG	58.05 57.72	XM_019350387.2	165	98.25
IL-8	CAAGATCATGTCCAGCAGATCC TCGTGAAAGGAACACGGTGA	58.86 59.54	NM_001279704.1	119	99.5
NF-κβ	TCGGTGTAGCAGGCTTTTGT GCTGCAGAGATGTGGGTGAT	59.89 60.11	XM_013277333.3	179	97.5
TLR-4	TTCGCAGCAGATGACCACTT CAATGCGCTCTGATGCTGTC	59.96 59.97	XM_005453840.4	82	99.35
IL-10	ATGAGCAGAAGGCCTGTCAC GCT CCCCAAATAGCCACACT	60.04 60.03	XM_013269189.3	84	96.84
Caspase-3	AGGTAGTCCACGGTGCAAAG CCAGGCAGGCAAACCTCTTA	59.97 59.96	NM_001282894.1	183	98.5
BAX	GCGGTAGGGGACATTATGGT CCT GGGTGTGATGCCATCTT	59.24 60.03	XM_019357746.2	195	97.45
BCL-2	GACTGTACCAGCCGGACTTC AAAGCAATAATCCGGCCCCA	60.11 60.03	XM_003437902.5	133	96.5

β-actin beta-actin, *IL-1β* interleukin 1β, *TGF-β* transforming growth factor-beta, *IL-6* interleukin-6, *IL-8* interleukin-8, *TLR2* Toll-like receptor 2, *IL-10* interleukin 10, *NF-kB* nuclear factor kappa B, *BAX* Bcl-2 associated X-protein, *BCL-2* B-cell lymphoma 2

(NTC) (RNAse free water only) and RT negative controls (RT) were included in each round of the qPCR assay (RT-). The relative fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ comparative technique (Schmittgen and Livak 2008); the expression of the target genes was adjusted to that of β-actin. The primers of all genes used in this study are illustrated in Table 2.

Immunohistochemical analysis

Paraffin sections of the tissues obtained from the intestine, liver, and kidney were stained for immunohistochemistry (IHC) after bacterial challenge, following the method described by Hsu et al. (1981). First, the tissue sections obtained from all experimental groups were dewaxed and hydrated. Then, they were stained following the manufacturer's protocol using IL-1β antibodies and NF-kB p65 antibodies (Cat. No. NB600–501 and NB100–82,088, respectively; Novus Biologicals, Centennial, CO, USA) following the method described by El-Araby et al. (2022). After the tissue sections were counterstained by hematoxylin, three immuno-labeled sections were analyzed per animal for each antigen ($n = 5$ animals per group).

Histomorphometric measurements and histopathological investigation

The intestinal samples were collected at two different points of the experiment. The first set of samples was collected before the bacterial challenge to evaluate the histological and morphometric measurements after the feeding period. The second set of samples was collected after the bacterial challenge. Three tissue sets were collected from the intestine, liver, and kidneys of the fish in all groups to examine the pathological alterations in these tissues following the challenge with *A. veronii*. The samples were fixed in 10% neutral buffered formalin, dehydrated in an ascending ethanol gradient, cleaned in xylene, and embedded in paraffin wax. Next, paraffin sections were cut into thin slices (5 µm thick) using a microtome (Leica RM 2155, England). The sections were prepared and stained with hematoxylin and eosin (H&E). The samples were examined, photographs were captured, and morphometric measurements were recorded under a microscope equipped with an AmScope digital camera (Suvarna et al. 2013). We captured 20 images per fish for morphometric measurements at ×4 and ×40 magnifications. All measurements (i.e., villus height and width, and muscular coat thickness) were recorded using the AmScopeToupView 3.7 software (AmScope, USA).

Statistical analysis

The Shapiro-Wilk test was conducted to determine whether the data followed a normal distribution. The differences among groups were determined by a two-way analysis of variance (ANOVA) in SPSS version 18 (SPSS, Chicago, IL, USA). The differences between groups were determined by Tukey's multiple range tests ($n = 9$/group). The variation in the data was presented as pooled SEM, and all differences among and between groups were considered to be statistically significant at $P \leq 0.05$.

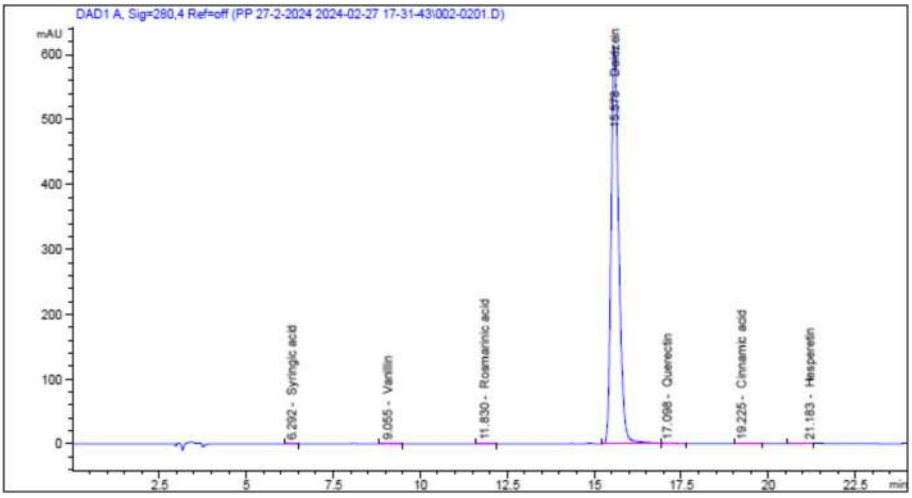

Fig. 2 HPLC analysis of LEO

Results

HPLC analysis

The major compounds determined by HPLC analysis of LEO were daidzein (6428.41 µg/mL), quercetin (27.04 µg/mL), hesperetin (9.02 µg/ml), rosmarinic acid (8.58 µg/mL), vanillin (3.18 µg/mL), and cinnamic acid (0.76 µg/mL) (Fig. 2).

Growth performance

The FBW, BWG, PER, and RGR were lower, and the FCR was higher in fish cultured under HSD compared to those cultured under LSD, irrespective of whether LEO was added ($P < 0.01$). At all stocking densities, the FBW, BWG, PER, and RGR increased significantly, and the FCR decreased in the fish that received 1% and 0.5% LEO ($P < 0.01$). The interaction between the stocking density and the level of LEO showed that only the FBW increased significantly in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups. The FBW was significantly lower in the HSD + LEO0 group than in the LSD + LEO0 group ($P < 0.01$) (Table 3).

Table 3 The effect of stocking density, LEO supplementation, and their interaction on the growth performance of the Nile tilapia

		IBW (g/fish)	FBW (g/fish)	TWG (g/fish)	FI (g/fish)	FCR	PER	SGR (%/day)
SD effect								
LSD		23.09	75.35	52.26	81	1.56	2.08	1.69
HSD		23.69	69.67	45.97	78	1.71	1.91	1.54
LEO effect								
0		23.65	67.03^{c}	43.38^{c}	77	1.78^{a}	1.82^{b}	1.49^{c}
0.5% LEO		23.48	74.09^{b}	50.60^{b}	80.5	1.60^{b}	2.03^{a}	1.64^{b}
1% LEO		23.04	76.42^{a}	53.36^{a}	81	1.52^{b}	2.13^{a}	1.71^{a}
SD × LEO								
LSD	LEO0	23	68.74^{d}	45.73	78.33	1.71	1.89	1.56
	LEO0.5	22.92	77^{b}	54.08	82.33	1.52	2.12	1.73
	LEO1	23.35	80.33^{a}	56.98	82.33	1.44	2.24	1.76
HSD	LEO0	24.30	65.33^{e}	41.03	75.67	1.84	1.75	1.41
	LEO0.5	24.04	71.17^{cd}	47.13	78.67	1.67	1.95	1.55
	LEO1	22.75	72.51^{c}	49.75	79.67	1.60	2.02	1.66
SEM		0.226	1.63	1.74	1.14	0.061	0.069	0.045
Two-way ANOVA: *P*-values								
Stocking density		0.092	< 0.01	< 0.01	0.07	< 0.01	< 0.01	< 0.01
LEO		0.342	< 0.01	< 0.01	0.107	< 0.01	< 0.01	< 0.01
Interaction		0.07	< 0.01	0.116	0.954	0.995	0.702	0.394

The data variation was represented as pooled *SEM*, standard error of the means. *LSD*, low stocking density; *HSD*, high stocking density; *IBW*, initial body weight; *FBW*, final body weight; *BWG*, body weight gain; *FI*, feed intake; *FCR*, feed conversion ratio; *PER*, protein efficiency ratio; *RGR*, relative growth rate. $^{a–d}$Means in the same column with different superscripts are significantly different ($P \leq 0.05$)

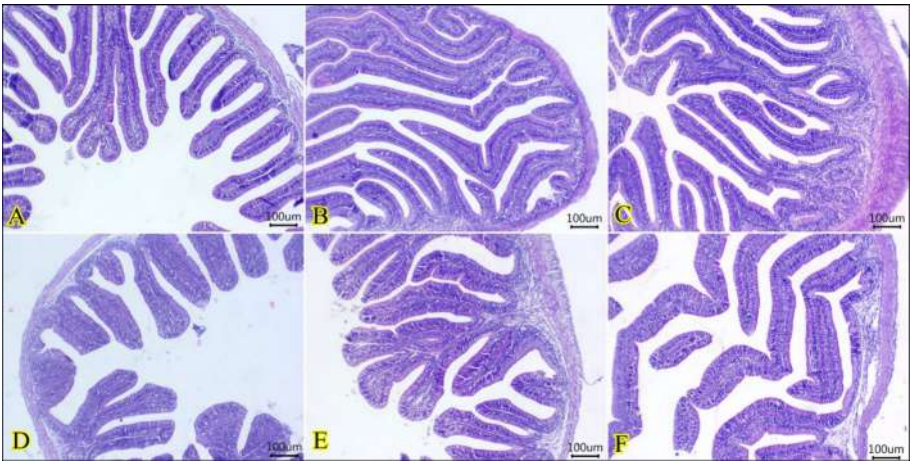

Fig. 3 Photomicrographs of H&E-stained sections (scale bar, 100 μm) of the intestine of Nile tilapia fish before they were infected. (A) Normal histology of mucosa, submucosa, and muscular layer in the LSD + LEO0 group. (B) Normal intestinal layers with shorter villi were found in the HSD + LEO0 group. (C) The architectural integrity of the mucosal layer was better, and the villus surface was greater in the LSD + LEO0.5 group. (D) Normal intestinal layers with a moderate number of branched villi in the HSD + LEO0.5 group. (E) The height of the villi and muscular thickness were considerably greater in the LSD + LEO1 group. (F) The villus structures were elongated and less branched in the HSD + LEO1 group

Histomorphometry of the intestine

The histological characteristics of the mucosa, submucosa, and muscular layer in the intestine were normal in the LSD + LEO0 group (Fig. 3A). The intestinal layers appeared normal with shorter villi in the HSD + LEO0 group (Fig. 3B). The architectural integrity of the mucosal layer was better, and the villus surface was greater in the LSD + LEO0.5 group (Fig. 3C). The intestinal layers appeared normal with branched ends in moderate villi in the HSD + LEO0.5 group (Fig. 3D). The villus height and muscular thickness were considerably greater in the LSD + LEO1 group than in the other groups treated with LEO (Fig. 3E). The HSD + LEO1 group showed to be elongated and less branched in the HSD + LEO1 group (Fig. 3F).

The morphometric measurements of the intestine are presented in Table 4. The stocking density did not significantly affect the height of the intestinal villi (VH) ($P > 0.05$). Irrespective of the effect of stocking density, the VH was significantly higher after treatment with 1% LEO compared to the VH in the absence of LEO ($P = 0.014$). The interaction between stocking density and LEO addition did not significantly affect the VH ($P > 0.05$). The stocking density and LEO addition, and their interaction did not significantly affect the intestinal villus width ($P > 0.05$). Irrespective of the effect of LEO, the muscular coat thickness (MT) was lower in the fish cultured under HSD compared to those cultured under LSD ($P = 0.016$). Irrespective of the effect of stocking density, the MT increased after 1% LEO was added ($P < 0.01$). The interaction revealed a significant increase in the MT in the LSD + LEO1 group compared to that in the LSD + LEO0 group ($P = 0.050$).

Table 4 The effect of stocking density, LEO supplementation, and their interaction on the morphometric measurements of the intestine of the Nile tilapia

		Villus height (μm)	Villus width (μm)	Musculosa thickness (μm)
SD effect				
LSD		438	81.2	67.1
HSD		351	69.4	46.3
LEO effect				
0		300[b]	73.3	36.5[b]
0.5% LEO		340[b]	70.0	54.8a[b]
1% LEO		545[a]	82.6	78.83[a]
SD × LEO				
LSD	LEO0	342	74.6	41.0[b]
	LEO0.5	343	81.0	56.6[b]
	LEO1	630	88.0	103.7[a]
HSD	LEO0	257	72.0	32.0[b]
	LEO0.5	337	59.0	53.0[b]
	LEO1	459	77.3	54.0[b]
SEM		38.8	5.98	6.31
Two-way ANOVA: *P*-values				
Stocking density		0.177	0.393	0.016
LEO		0.014	0.729	< 0.01
Interaction		0.55	0.83	0.050

The data variation was represented as pooled *SEM*, the standard error of the means. *LSD*, low stocking density; *HSD*, high stocking density. [a,b]Means in the same column with different superscripts are significantly different ($P \leq 0.05$)

Digestive enzyme activity

The activities of amylase and lipase were significantly lower in the fish cultured under HSD compared to that in the fish cultured under LSD ($P < 0.01$). Regardless of the effect of stocking density, LEO increased the amylase and lipase activity in a dose-dependent manner ($P < 0.01$). The interaction between the stocking density and LEO level significantly increased the lipase activity in the LSD + LEO1 and LSD + LEO0.5 groups, followed by the HSD + LEO1 and HSD + LEO0.5 groups, compared to the lipase activity recorded in the LSD + LEO0 and HSD + LEO0 groups ($P < 0.01$). The interaction between the stocking density and LEO level did not significantly affect amylase activity ($P > 0.05$) (Table 5).

Blood biochemical parameters

The serum levels of total protein, albumin, and globulin were reduced, while the albumin/globulin ratio was higher in the fish cultured under HSD compared to the ratio in the fish cultured under LSD ($P < 0.05$). Regardless of the effect of stocking density, adding LEO increased the serum levels of total protein, albumin, and globulin, but decreased the albumin/globulin ratio in a dose-dependent manner ($P < 0.05$). The interaction

Table 5 The effect of stocking density, LEO supplementation, and their interaction on the blood proteinogram and intestinal digestive enzymes activities of the Nile tilapia

		Amylase (U/mg)	Lipase (ng/mg)	TP (g/dL)	ALB (g/dL)	GLB (g/dL)	A/G ratio
SD effect							
LSD		15.45	11.98	8.39	3.81	4.590	0.821
HSD		11.84	7.40	4.73	2.21	2.41	0.983
LEO effect							
0		9.15^{c}	4.42^{c}	2.97^{c}	1.46^{c}	1.51^{c}	1.05^{a}
0.5% LEO		14.54^{b}	11.04^{b}	7.04^{b}	3.27^{b}	3.77^{b}	0.820^{b}
1% LEO		17.25^{a}	13.62^{a}	9.66^{a}	4.28^{a}	5.21^{a}	0.828^{b}
SD × LEO							
LSD	LEO0	9.60	5.16^{cd}	3.59^{de}	1.57^{d}	2.02^{de}	0.78^{b}
	LEO0.5	16.97	14.12^{a}	9.12^{b}	4.20^{b}	4.92^{b}	0.85^{b}
	LEO1	19.80	16.68^{a}	12.47^{a}	5.65^{a}	6.83^{a}	0.83^{b}
HSD	LEO0	8.69	3.67^{d}	2.36^{e}	1.35^{d}	1.00^{e}	1.34^{a}
	LEO0.5	12.13	7.96^{bc}	4.97^{d}	2.35^{cd}	2.62^{cd}	0.79^{b}
	LEO1	14.70	10.57^{b}	6.86^{c}	2.93^{c}	3.60^{c}	0.82^{b}
SEM		0.999	1.14	0.839	0.374	0.474	0.056
Two-way ANOVA: *P*-values							
Stocking density		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.039
LEO		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.027
Interaction		0.052	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

The data variation was represented as pooled *SEM*, standard error of the means. *LSD*, low stocking density; *HSD*, high stocking density. $^{a–d}$Means in the same column with different superscripts are significantly different ($P \leq 0.05$)

between the stocking density and LEO level significantly increased the serum levels of total protein, albumin, and globulin in the LSD + LEO1 and LSD + LEO0.5 groups, followed by the HSD + LEO1 and HSD + LEO0.5 groups, compared to that in the LSD + LEO0 and HSD + LEO0 groups ($P < 0.01$). The albumin/globulin ratio was significantly higher in the HSD + LEO0 group than in other experimental groups ($P < 0.01$) (Table 5).

The serum levels of ALT, ALP, creatinine, urea, and glucose were significantly higher in the fish cultured under HSD than in the fish cultured under LSD ($P < 0.05$). The growth hormone level was significantly lower in the serum of fish cultured under HSD than in the serum of fish cultured under LSD ($P < 0.01$), whereas the serum level of AST was not significantly different between the LSD and HSD groups ($P > 0.05$). Regardless of the effect of stocking density, adding LEO decreased the serum levels of ALT, ALP, creatinine, urea, and glucose, and increased the GH level in a dose-dependent manner ($P < 0.01$). However, LEO did not significantly affect the AST level ($P > 0.05$). The interaction between the stocking density and LEO level significantly decreased the serum levels of ALP and creatinine and increased the GH level in the LSD + LEO1 and LSD + LEO0.5 groups, followed by the HSD + LEO1 and HSD + LEO0.5 groups, compared to that in the LSD + LEO0 and HSD + LEO0 groups ($P < 0.01$). The glucose level was significantly lower in the LSD + LEO1 and LSD + LEO0.5 groups than the

Table 6 The effect of stocking density, LEO supplementation, and their interaction on the blood biochemical parameters of the Nile tilapia

		ALT (U/L)	AST (U/L)	ALP (U/L)	Creatinine (mg/dL)	Urea (mg/dL)	Glucose (mg/dL)	GH (pg/dL)
SD effect								
LSD		7.86	70.99	59.09	0.510	11.15	76.10	45.82
HSD		13.96	78.20	77.81	0.613	18.62	85.59	41.04
LEO effect								
0		19.09^{a}	87.26	91.74^{a}	0.710^{a}	24.32^{a}	93.57^{a}	37.18^{c}
0.5% LEO		8.22^{b}	73.37	60.72^{b}	0.510^{b}	11.85^{b}	79.65^{ab}	45.24^{b}
1% LEO		5.41^{c}	63.15	52.84^{c}	0.465^{c}	8.49^{c}	69.31^{b}	47.88^{a}
SD × LEO								
LSD	LEO0	16.77	95.30	88.70^{b}	0.670^{b}	21.47	97.32^{a}	39.17^{e}
	LEO0.5	4.76	65.28	48.22^{e}	0.452^{e}	7.44	68.67^{bc}	47.64^{b}
	LEO1	2.06	52.42	40.36^{f}	0.411^{f}	4.55	62.32^{c}	50.66^{a}
HSD	LEO0	21.43	79.23	94.79^{a}	0.752^{a}	27.18	89.83^{ab}	35.21^{f}
	LEO0.5	11.70	81.47	73.33^{c}	0.573^{c}	16.27	90.65^{ab}	42.84^{d}
	LEO1	8.77	73.90	65.32^{d}	0.520^{d}	12.43	76.29^{abc}	45.10^{c}
SEM		1.62	5.67	4.78	0.028	1.90	3.55	1.25
Two-way ANOVA: *P*-values								
Stocking density		< 0.01	0.528	< 0.01	< 0.01	< 0.01	0.047	< 0.01
LEO		< 0.01	0.224	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Interaction		0.082	0.358	< 0.01	0.012	0.057	0.041	0.022

The data variation was represented as pooled *SEM*, the standard error of the means. *LSD*, low stocking density; *HSD*, high stocking density. $^{a–f}$Means in the same column with different superscripts are significantly different ($P \leq 0.05$)

Table 7 The effect of stocking density, LEO supplementation, and their interaction on the immune/antioxidant responses of the Nile tilapia

		LYZ (μg/mL)	NO (mg/mL)	C3 (mg/dL)	CAT (U/g)	SOD (U/g)	GST (U/g)	MDA (mmol/g)
SD effect								
LSD		23.72	35.06	56.66	17.92	65.48	15.98	10.33
HSD		19.77	31.47	45.78	11.86	57.91	10.27	18.63
LEO effect								
0		18.09^{c}	29.38^{c}	40.66^{c}	8.84^{c}	53.99^{c}	6.96^{c}	20.14^{a}
0.5% LEO		22.50^{b}	33.93^{b}	54.00^{b}	15.81^{b}	63.74^{b}	14.90^{b}	13.14^{ab}
1% LEO		24.66^{a}	36.48^{a}	59.00^{a}	20.03^{a}	67.35^{a}	17.52^{a}	10.15^{b}
SD × LEO								
LSD	LEO0	19.34^{e}	30.61^{d}	43.00^{e}	9.93^{de}	56.67^{e}	8.26^{e}	14.12
	LEO0.5	24.39^{b}	36.15^{b}	61.00^{b}	19.55^{b}	67.95^{b}	18.51^{b}	9.83
	LEO1	27.44^{a}	38.43^{a}	66.00^{a}	24.29^{a}	71.83^{a}	21.17^{a}	7.04
HSD	LEO0	16.84^{f}	28.16^{e}	38.33^{f}	7.77^{e}	51.33^{f}	5.66^{f}	26.16
	LEO0.5	20.61^{d}	31.72^{d}	47.00^{d}	12.07^{d}	59.53^{d}	11.29^{d}	16.45
	LEO1	21.88^{c}	34.53^{c}	52.00^{c}	15.77^{c}	62.87^{c}	13.86^{c}	13.27
SEM		0.834	0.844	2.36	1.39	1.66	1.32	1.73
Two-way ANOVA: *P*-values								
Stocking density		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
LEO		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Interaction		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	0.050

The data variation was represented as pooled *SEM*, standard error of the means. *LSD*, low stocking density; *HSD*, high stocking density. [a–f]Means in the same column with different superscripts are significantly different ($P \leq 0.05$)

LSD + LEO0 group ($P < 0.01$). The levels of AST, ALT, and urea were not significantly affected by the interaction term ($P > 0.05$) (Table 6).

Immune/antioxidant status

Irrespective of whether LEO was added, the serum levels of LYZ, NO, and C3 were lower in the HSD group than in the LSD group ($P < 0.01$). Irrespective of the stocking density, these immune indices increased significantly with an increase in the LEO level compared to their corresponding values in the absence of LEO ($P < 0.01$). The interaction between the stocking density and LEO level significantly increased the serum levels of LYZ, NO, and C3 in the LSD + LEO1 and LSD + LEO0.5 groups, followed by the HSD + LEO1 and HSD + LEO0.5 groups, compared to that in the LSD + LEO0 and HSD + LEO0 groups ($P < 0.01$) (Table 7).

Fish cultured under HSD showed lower antioxidant activity, indicated by lower CAT, SOD, and GST levels and higher MDA levels, than those cultured under LSD ($P < 0.01$). Regardless of the stocking density, the addition of LEO significantly increased the levels of the antioxidant indicators (CAT, SOD, and GST) and decreased the MDA levels in a dose-dependent manner ($P < 0.01$). The interaction between the stocking density and LEO level significantly increased the serum levels of CAT, SOD, and GST in the LSD + LEO1 and

LSD + LEO0.5 groups, followed by the HSD + LEO1 and HSD + LEO0.5 groups, compared to that in the LSD + LEO0 and HSD + LEO0 groups ($P < 0.01$). The MDA levels were not significantly different between the experimental groups ($P = 0.05$) (Table 7).

Survivability rates and clinical signs following the *A. veronii* challenge test

After the fish were challenged with *A. veronii*, they showed several clinical signs of infection, including sluggish swimming, cutaneous hemorrhage on various parts of the body, different degrees of fin rot, and localized patches of scale loss. The LSD + LEO1 group showed the highest survivability rate (86.66%) among all challenged fish groups, followed by the LSD + LEO0.5 group (83.33%); the LSD + LEO0 group showed a survivability rate of 66.66%. The survivability rate (53.33%) was the lowest in the HSD + LEO0 group, but it was significantly higher in the HSD + LEO1 (76.66%) and HSD + LEO0.5 (73.33%) groups.

Expression level of the mRNAs of immune-related genes

The level of expression of *IL-1β*, *IL-6*, *IL-8*, *NF-kB*, and *TLR-4 B* was significantly higher in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups than in the LSD + LEO0 group ($P < 0.01$). However, the level of expression of *IL-6* and *NF-kB* was lower in the

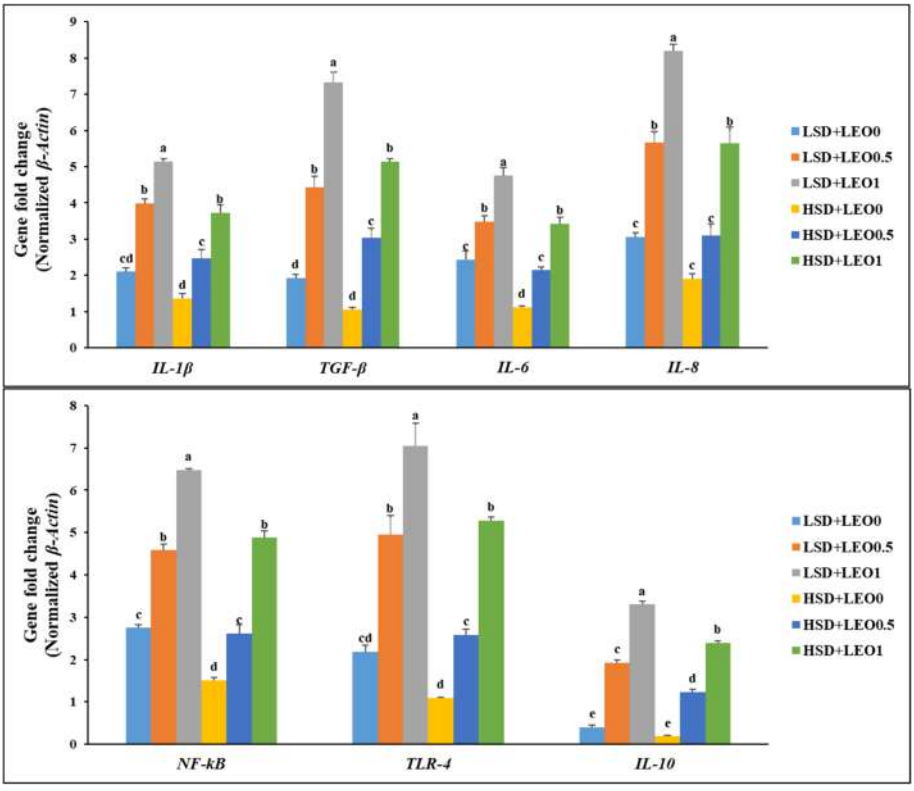

Fig. 4 Quantitative real-time PCR analysis of the expression of immune-related genes (*IL-1β*, *TGF-β*, *IL-6*, *IL-8*, *NF-kB*, *TLR-4*, and *IL-10*) of the Nile tilapia challenged with *A. veronii* ($n = 9$) in each experimental group. The bars with different letters (a–e) indicate significant differences ($P < 0.05$)

HSD + LEO0 group compared to that in the LSD + LEO0 group ($P < 0.01$). The level of expression of *TGF-β* and *IL-10* was significantly higher in the fish that were administered 1% and 0.5% LEO and cultured under LSD and HSD compared to that recorded in the fish in the LSD + LEO0 group ($P < 0.01$) (Fig. 4).

Expression level of the mRNAs of apoptosis-related genes

The level of expression of *caspase 3* was the highest in the HSD + LEO0 group, followed by the HSD + LEO0.5 group, whereas its level of expression was lower in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups compared to that in the LSD + LEO0 group ($P < 0.01$). The level of expression of *BAX* was higher in the HSD + LEO0 group and lower in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups compared to that in the LSD + LEO0 group ($P < 0.01$). The level of expression of *BCL-2* was lower in the HSD + LEO0 group and higher in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups compared to that in the LSD + LEO0 group ($P < 0.01$). The level of expression of *BAX/BCL-2* was higher in the HSD + LEO0 group and lower in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups compared to that in the LSD + LEO0 group ($P < 0.01$) (Fig. 5).

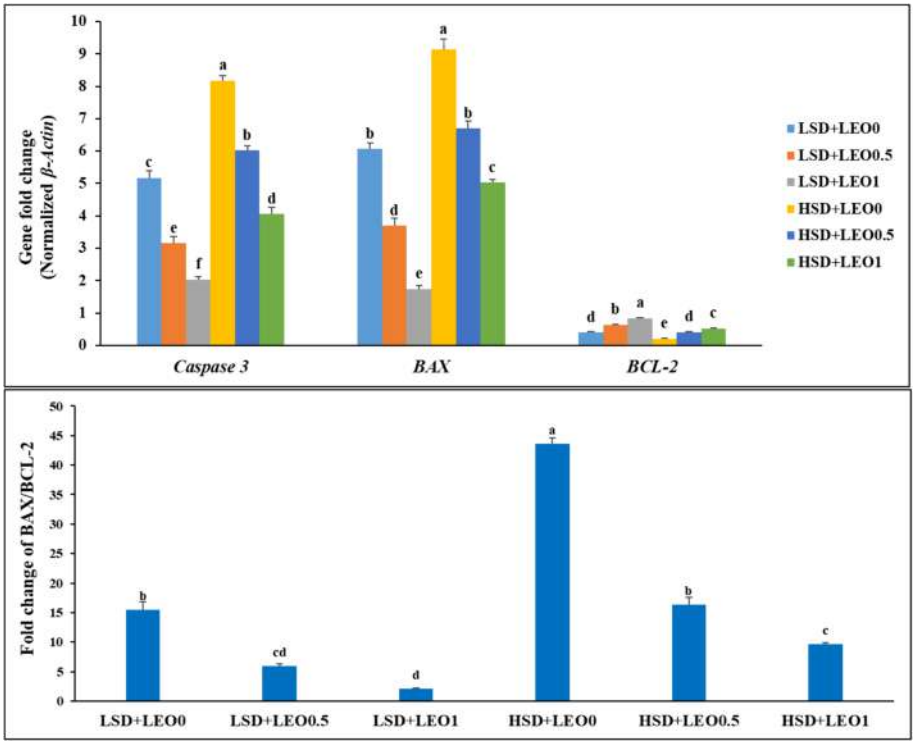

Fig. 5 Quantitative real-time PCR analysis of the expression of apoptosis-related genes (*caspase 3*, *BAX*, *BCL-2*, and BAX/BCL-2) in the Nile tilapia challenged with *A. veronii* ($n = 9$) in the different experimental groups. The bars with different letters (a–f) indicate significant differences ($P < 0.05$)

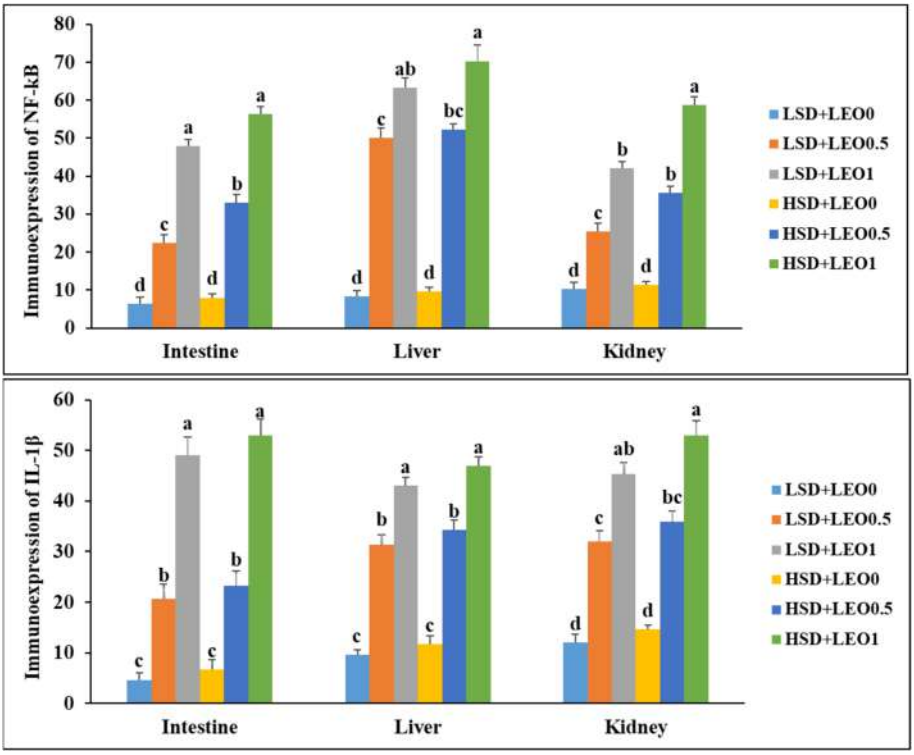

Fig. 6 Analytical data of the immunostained *NF-kB* and *LI-1β*-positive cells in the tissues of the Nile tilapia challenged with *A. veronii*. Bars with different letters (a–d) indicate significant differences ($P < 0.05$)

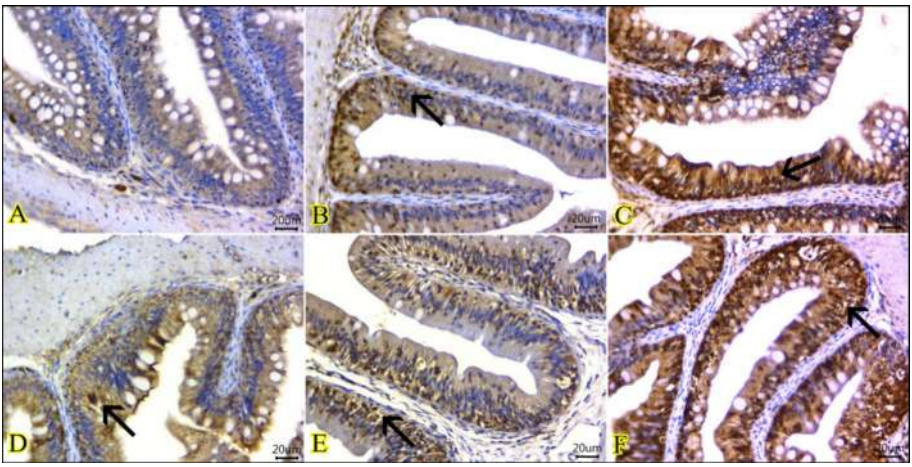

Fig. 7 Representative photomicrographs of the intestinal sections of *A. veronii*-challenged Nile tilapia immunostained for NF-κβ P65 showed non-observable expressed cells in the LSD + LEO0 group (A). The columnar mucosal epithelium in the LSD + LEO0.5 group was stained mildly (B). A moderate number of cells were stained in the LSD + LEO1 group (C). Few cells were immunolabeled in the HSD + LEO0 group (D). The cells in the HSD + LEO0.5 group were stained mildly (E). The cells in the HSD + LEO1 group were stained less intensely (F). IHC counterstaining was performed with Mayer's hematoxylin; scale bar, 20 µm

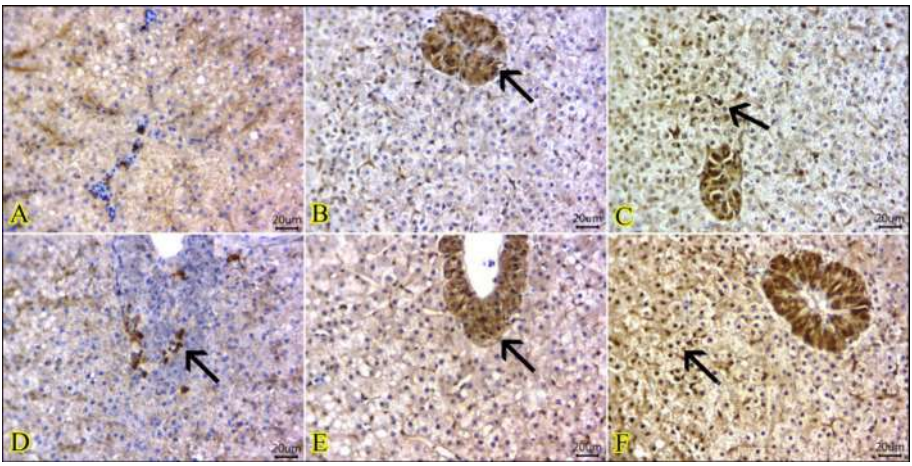

Fig. 8 Representative photomicrographs of the liver sections of *A. veronii*-challenged Nile tilapia immunostained for NF-κβ P65 showed a few immunolabeled cells in the LSD + LEO0 group (A). The nuclei of a moderate number of cells in the LSD + LEO0.5 group were stained (B). The nuclei of many cells in the LSD + LEO1 group were stained (C) in hepatic and pancreatic cells. Only a few cells in the HSD + LEO0 group were stained (D). A moderate number of cells in the HSD + LEO0.5 group were immunolabeled (E). Many cells in the HSD + LEO1 group were immunostained (F). IHC counterstaining was performed with Mayer's hematoxylin; scale bar, 20 μm

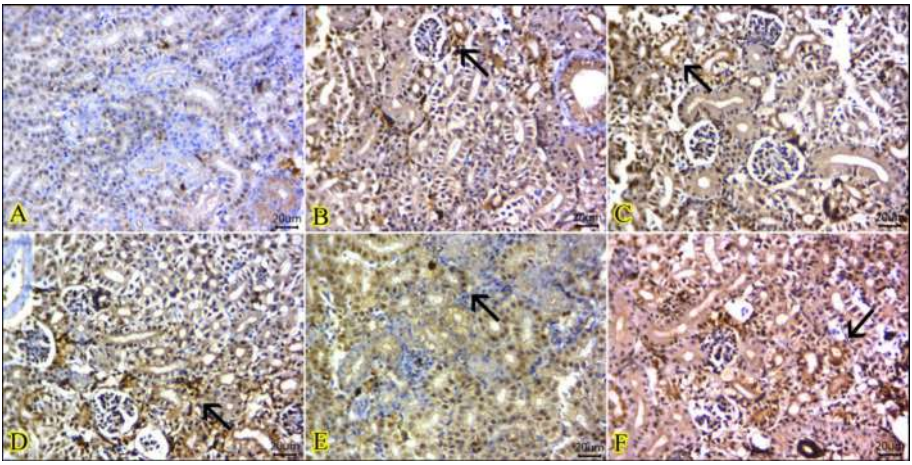

Fig. 9 Representative photomicrographs of kidney sections of *A. veronii*-challenged Nile tilapia immunostained for NF-κβ P65 showed focally distributed positive immunolabeled cells in the LSD + LEO0 group (A). Only a few positive cells were found in the LSD + LEO0.5 group (B). Diffusely distributed nuclear expression in the renal tubular epithelium in the LSD + LEO1 group (C). Only a few cells were labeled in the epithelium of the renal tubules in the HSD + LEO0 group (D). A moderate number of cells in the HSD + LEO0.5 group were stained (E). Many cells in the HSD + LEO1 group were immunolabeled (F). IHC counterstaining was performed with Mayer's hematoxylin; scale bar, 20 μm

Immunohistochemistry analysis

The immunostaining of the tissues from the intestine, liver, and kidneys showed increased expression of *NF-kB* in the HSD + LEO1, LSD + LEO1, HSD + LEO0.5, and LSD + LEO0.5 groups, with the percentages of immunostained cells in the intestine being 56%, 48%, 33%, and 22%, respectively, compared to 8% and 6% in the HSD + LEO0 and LSD + LEO0 groups, respectively (Figs. 6 and 7). In the liver, the percentages of the immunostained cells were 70%, 63%, 52%, 50%, 10%, and 8% for HSD + LEO1, LSD + LEO1, HSD + LEO0.5, LSD + LEO0.5, HSD + LEO0, and LSD + LEO0, respectively (Figs. 6 and 8). In the kidney, the percentages of the immunostained cells were 58%, 42%, 35%, 25%, 11%, and 10% for HSD + LEO1, LSD + LEO1, HSD + LEO0.5, LSD + LEO0.5, HSD + LEO0, and LSD + LEO0 groups, respectively (Figs. 6 and 9).

The immunostaining of the intestine, liver, and kidney tissues showed higher expression of *IL-1β* in the HSD + LEO1, LSD + LEO1, HSD + LEO0.5, and LSD + LEO0.5 groups, with the percentages of immunostained cells in the intestine being 53%, 49%, 23%, and 21%, respectively, compared to 7% and 5% in the HSD + LEO0 and LSD + LEO0 groups, respectively (Figs. 6 and 10). In the liver, the percentages of the immunostained cells were 475, 43%, 34%, 31%, 11%, and 9% in the HSD + LEO1, LSD + LEO1, HSD + LEO0.5, LSD + LEO0.5, HSD + LEO0, and LSD + LEO0 groups, respectively (Figs. 6 and 11). In the kidney, the percentages of the immunostained cells were 53%, 45%, 36%, 32%, 15%, and 12% in the HSD + LEO1, LSD + LEO1, HSD + LEO0.5, LSD + LEO0.5, HSD + LEO0, and LSD + LEO0 groups, respectively (Figs. 6 and 12).

Histopathological findings following challenge with *A. veronii*

Short villus architectures with edematous lamina propria were found in the intestines of the fish in the LSD + LEO0 group (Fig. 13A). The villus epithelium was well-maintained, prominent lymphoid aggregates beside lymphocytosis were detected, and acidophilic

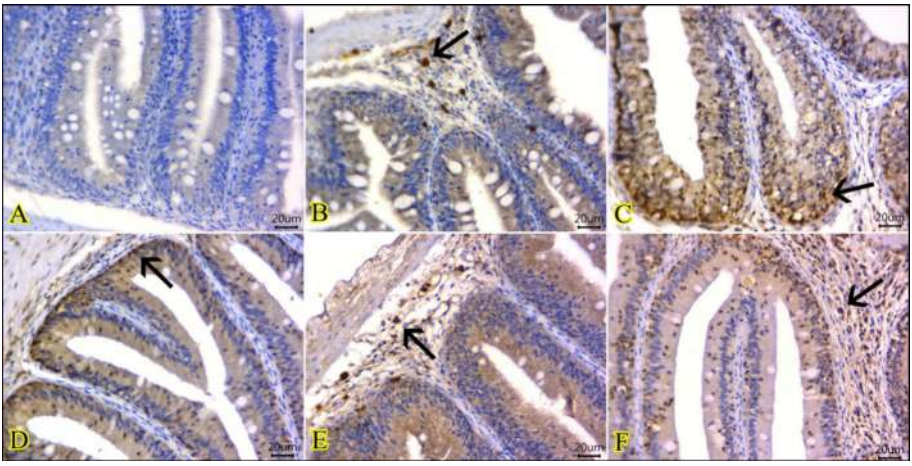

Fig. 10 Representative photomicrographs of the intestinal sections of *A. veronii*-challenged Nile tilapia immunostained for IL-1β showed no expression in the LSD + LEO0 group (A). Fewer immune cells in the LSD + LEO0.5 group expressed IL-1β (B). Many cells in the LSD + LEO1 group were immunolabeled (C). Few cells in the HSD + LEO1 group were immunoreactive (D). A moderate number of cells in the HSD + LEO0.5 group expressed IL-1β (E). The inflammatory cells in the HSD + LEO1 group expressed high levels of IL-1β (F). IHC counterstaining was performed with Mayer's hematoxylin; scale bar, 20 μm

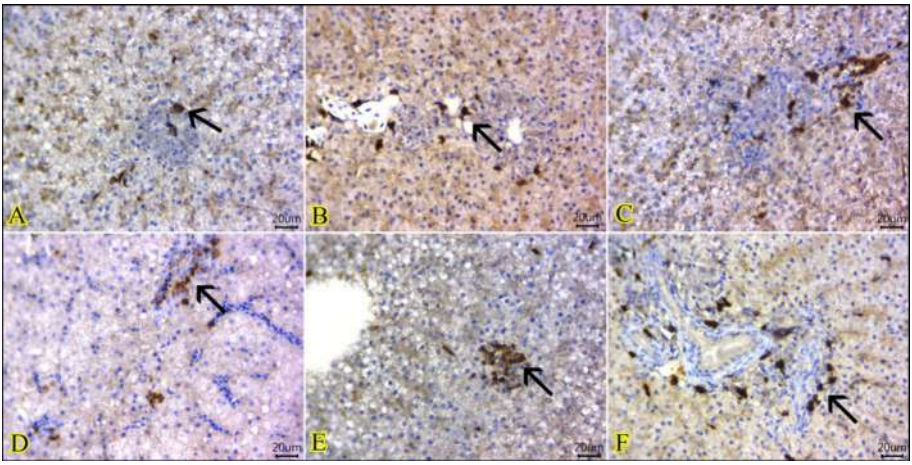

Fig. 11 Representative photomicrographs of liver sections of *A. veronii*-challenged Nile tilapia immunostained for IL-1β showed a few stained cells in the LSD + LEO0 group (A). The inflammatory cells in the LSD + LEO0.5 group were mildly immunolabeled (B). Several positive immunoreactive inflammatory cells were found in the LSD + LEO1 group (C). A few cells in the HSD + LEO0 group were labeled (D). A moderate number of reactive cells were found in the HSD + LEO0.5 group (D). Many cells in the HSD + LEO1 group were immunoreactive (F). IHC counterstaining was performed with Mayer's hematoxylin; scale bar, 20 μm

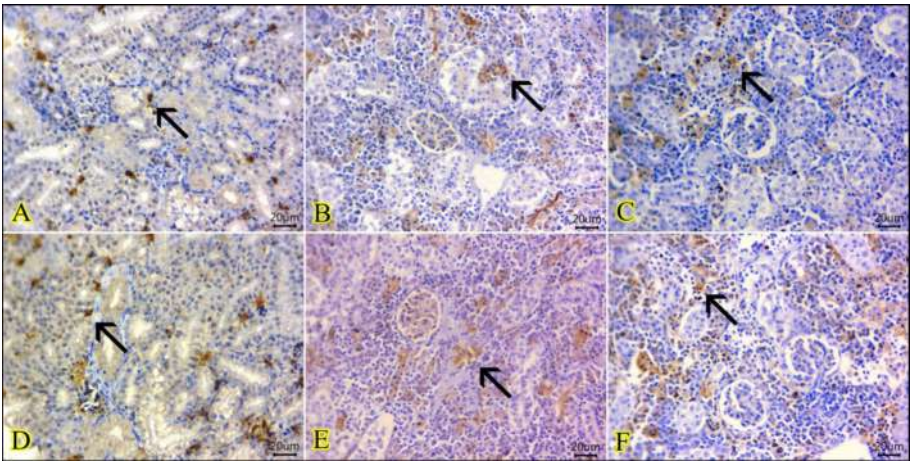

Fig. 12 Representative photomicrographs of kidney sections of *A. veronii*-challenged Nile tilapia immunostained for IL-1β showed unicellular positively labeled cells in the LSD + LEO0 group (A). A moderate number of positive cells were found in the LSD + LEO0.5 group (B). The inflammatory cells in the LSD + LEO1 group showed high levels of IL-1β protein (C). Only a few cells in the HSD + LEO0 group were immunostained (D). A moderate number of cells in the HSD + LEO0.5 group were immunoreactive (E). The inflammatory cells in the HSD + LEO1 group showed high expression of IL-1β protein (F). IHC counterstaining was performed with Mayer's hematoxylin; scale bar, 20 μm

granulocytes were found in the submucosal layer in the LSD + LEO0.5 group (Fig. 13B and C). The structures of the intestinal layers were preserved, and abundant acidophilic granulocytes were found in the submucosal layer of the LSD + LEO1 group (Fig. 13D and

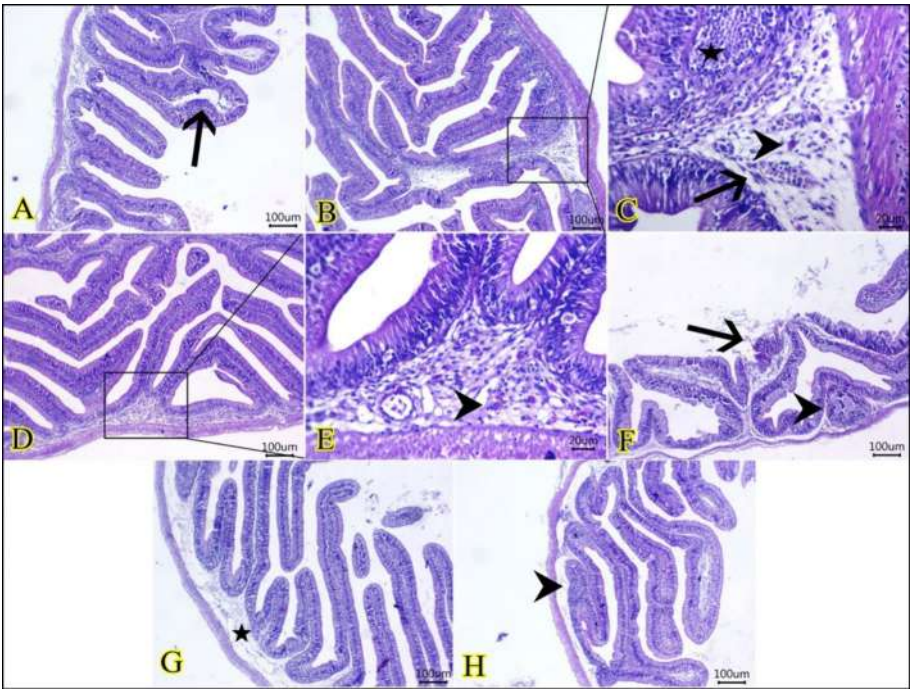

Fig. 13 Photomicrographs of H&E-stained sections from the intestine of *A. veronii*-challenged Nile tilapia. (A) In the LSD + LEO0 group, short villus architectures were found with edematous lamina propria (arrowhead). (B and C) In the LSD + LEO0.5 group, villus epithelium was maintained, lymphoid aggregates were prominent (star) beside lymphocytosis (arrow), and acidophilic granulocytes were found within the submucosal layer (arrowhead). (D and E) In the LSD + LEO1 group, the structures of intestinal layers were preserved, and abundant acidophilic granulocytes were present within the submucosal layer. (F) In the HSD + LEO0 group, the epithelium underwent focal necrosis (arrow), and the submucosal lymphoid elements were depleted (arrowhead). (G) In the HSD + LEO0.5 group, the morphology of intestinal layers was normal, and submucosal edema was present (star). (H) In the HSD + LEO1 group, mild submucosal edema and prominent lymphoid populations (arrowhead) with broad villus ends were recorded

E). The mucosal epithelium underwent focal necrosis, and the submucosal lymphoid elements were depleted in the HSD + LEO0 group (Fig. 13F). The morphology of the intestinal layers was normal, and submucosal edema was recorded in the HSD + LEO0.5 group (Fig. 12G). However, mild submucosal edema with broad villus ends and prominent lymphoid populations was found in the HSD + LEO1 group (Fig. 13H).

In the LSD + LEO0 group, a moderate area of the hepatic parenchyma showed steatosis, and focal round cell aggregates were found in the pancreas (Fig. 14A). In the LSD + LEO 0.5 group, mild degenerative changes were found in few hepatocytes and the structures of pancreatic tissue were preserved (Fig. 14B). In the LSD + LEO 1 group, the integrity of most hepatocytes and pancreatic acini were maintained (Fig. 14C). In the HSD + LEO0 group, the sinusoids were congested and fatty changes were recorded in a large area (Fig. 14D). In the LSD + LEO 0.5 group, randomly distributed fatty degenerated cells and normal pancreatic tissue were present (Fig. 14E). However, in the LSD + LEO1 group, only a few fatty degenerated cells were present and the portal vein was congested (Fig. 14F).

In the LSD + LEO0 group, the periglomerular areas showed focal necrosis and the renal tubular epithelium showed hydropic degeneration (Fig. 15A). In the LSD + LEO0.5 group,

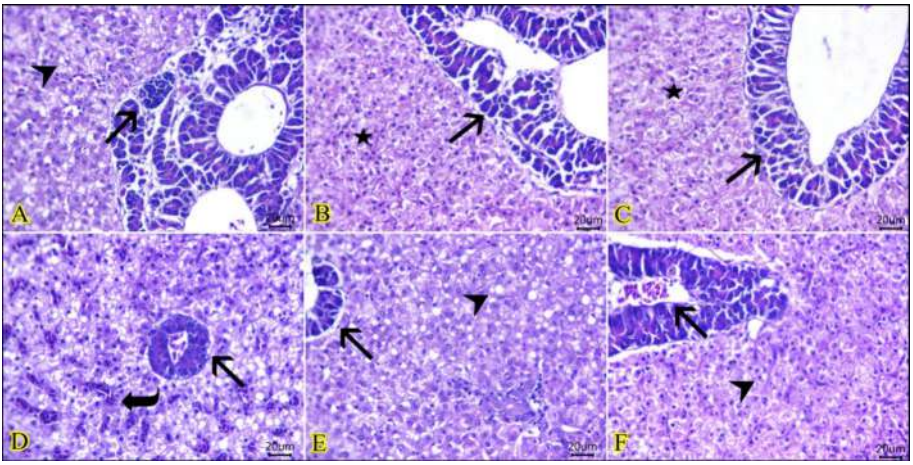

Fig. 14 Photomicrographs of H&E-stained sections (scale bar, 20 µm) from the hepatopancreas of *A. veronii*-challenged Nile tilapia. (A) In the LSD + LEO0 group, a moderate area of hepatic parenchyma showed steatosis (arrowhead), and focal round cell aggregates were found in the pancreatic areas (arrow). (B) In the LSD + LEO0.5 group, a few hepatocytes showed mild degenerative changes (star), but the pancreatic tissue (arrow) structures were preserved. (C) In the LSD + LEO1 group, the integrity of most hepatocytes (star) and pancreatic acini (arrow) was maintained. (D) In the HSD + LEO0 group, congested sinusoids (curved arrow) and a wide area of fatty change (arrowhead) were recorded. (E) In the HSD + LEO0.5 group, fatty degenerated cells were randomly distributed (arrowhead), and the pancreatic tissue appeared normal (arrow). (F) In the HSD + LEO1 group, a few fatty degenerated cells (arrowhead) and a congested portal vein (arrow) were recorded

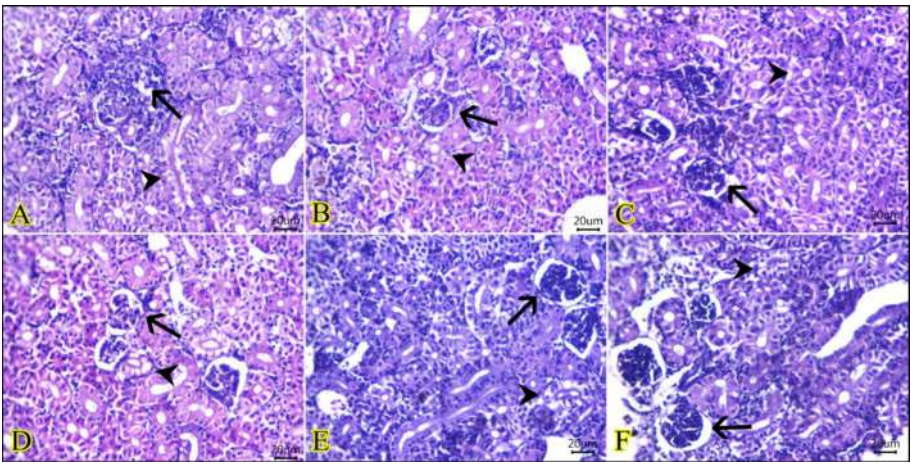

Fig. 15 Photomicrographs of H&E-stained sections (scale bar, 20 µm) from the kidney of *A. veronii*-challenged Nile tilapia. (A) In the LSD + LEO0 group, periglomerular areas showed focal necrosis (arrow), and the renal tubular epithelium showed hydropic degeneration (arrowhead). (B) In the LSD + LEO0.5 group, a few tubules were degenerated (arrowhead), and the glomerular corpuscles appeared normal (arrow). (C) In the LSD + LEO1 group, the renal tubular epithelium (arrowhead), glomeruli (arrow), and stromal structures were preserved. (D) In the HSD + LEO0 group, the glomeruli underwent atrophy (arrow), and a moderate number of renal tubules underwent necrosis (arrowhead). (E) In the HSD + LEO0.5 group, a few necrotic tubules with pyknotic nuclei (arrowhead) and normal glomerular corpuscles (arrow) were present. (F) In the HSD + LEO1 group, a few tubules (arrowhead) and glomeruli (arrow) were degenerated

a few tubules degenerated but the glomerular corpuscles were normal (Fig. 15B). Moreover, in the LSD + LEO1 group (Fig. 15C), the renal tubular epithelium, glomeruli, and stromal structures were preserved. In the HSD + LEO0 group, glomeruli were atrophied, and a moderate number of renal tubules underwent necrosis (Fig. 15D). In the HSD + LEO0.5 group, a few tubules underwent necrosis, and the nuclei underwent pyknosis, but the glomerular corpuscles were normal (Fig. 15E). In the HSD + LEO1 group, a few tubules underwent degeneration, but the glomeruli appeared to be normal (Fig. 15F).

Discussion

Stocking density is among the most important factors affecting fish health. Rearing fish under high stocking densities induces chronic stress (Ahmed et al. 2021). Stress factors alter the hormone levels in the hypothalamus-pituitary-interrenal axis and the hypothalamus-sympathetic-chromaffin (HSC) axis. These changes elicit various physiological responses that help coordinate and maintain homeostasis (Souza et al. 2019). Various activities in aquaculture, such as handling, transportation, and stocking density, stimulate stress systems, resulting in adverse effects on the physiological functions of fish, including growth, immunity, and reproduction. Thus, researchers have investigated the use of plant-derived essential oils (EOs) in aquaculture studies because they have several beneficial properties, such as anesthetic, antioxidant, and antimicrobial properties, which can decrease the changes in biochemical and endocrine functions, thus improving the health of fish. We found that the growth performance of fish decreased when cultured under HSD. The final body weight of fish cultured under LSD and HSD was higher when LEO was added to the diet compared to the final body weight under a non-supplemented diet. This result indicated that LEO reduced the adverse effect of HSD on fish growth. These positive effects of LEO occurred probably because the immunity and antioxidant status of fish increased after LEO was added. The secretion of growth hormone increased in the fish that were administered LEO and cultured under LSD and HSD. Growth hormones control the growth and the endocrine factors of fish related to the feeding status (Reinecke 2010; Wood et al. 2005). Growth hormones secreted by the pituitary gland promote the release of IGF-1 from the liver, which stimulates somatic growth (Björnsson et al. 2002). The increase in growth promoted by LEO may also be due to the enhancement of amylase and lipase activity (reported in this study) and an increase in villus height and muscular coat thickness, especially after treatment with 1% LEO, which improved nutrient digestibility and availability. Volatile components in essential oils act through specific odorant receptors by stimulating the central nervous system to manage energy expenditure, balance lipolysis and lipogenesis, and control appetite (De Blasio et al. 2021; Shen et al. 2005). The growth performance of fish improved after LEO was added probably because of the presence of acetic acid linalool ester (32.24%) and α-linalool (16.06%), which have appetizing properties and promote the functions of the digestive system (Cabuk et al. 2003). Moreover, daidzein (4′, 7-dihydroxyisoflavone), the major component of LEO, is a naturally occurring isoflavonic phytoestrogen belonging to the non-steroidal estrogens (Cassidy 2003), which has been reported to improve growth of stressed fish (Furna 2019). The improved growth of LEO-fed fish may be due to LEO content from quercetin that has been reported to improve the growth performance of Nile tilapia (Zhai and Liu 2013), snakehead fish, *Channa argus* (Kong et al. 2022), and blunt snout bream, *Megalobrama amblycephala* (Jia et al. 2019).

In this study, fish reared under high stocking densities had higher serum levels of ALT, ALP, creatinine, urea, and glucose. However, adding LEO to the diet reduced the serum levels of ALP, creatinine, and glucose in fish cultured under LSD and HSD, especially when the dose of LEO was 1%. LEO decreased the serum levels of AST and ALT (liver function enzymes that act as markers for liver damage), which indicated that LEO has hyperprotective effects (Selmi et al. 2015). The hepatoprotective effect of LEO might be associated with the antioxidant properties of the active compounds, such as eucalyptol, αpinene, α-terpineol, αlinalool, 9,12,15-octadecatrienoic acid, and arachidonic acid (Bouzenna et al. 2017; Seol and Kim 2016; Hsouna et al. 2022; Złotek et al. 2017; Al-Gara et al. 2019). Under stress, we found that the glucose levels in the fish were higher, which was similar to the findings of other studies on common carp under stress (Hosseini and Hoseini 2012; Hoseini et al. 2019). Such alterations in blood glucose levels are adaptive responses that provide the energy required under stressful conditions (Barton 2002). Dietary LEO has anti-stress effects, indicated by a reduction in blood glucose levels after its administration (Yousefi et al. 2020). The histopathological analysis of fish tissues (intestine, liver, and kidney) in this study confirmed that LEO had anti-stress effects, considering that 1% LEO strongly prevented tissue damage in fish cultured under high stocking densities. The anti-stress effects of LEO may be due to the presence of cineole, which is known to alleviate stress in various fish species (Mirghaed et al. 2018; Hoseini et al. 2018).

Studies have reported the negative effect of stress on the immune system (Tort 2011). The immune system is influenced by various phytochemicals, such as alkaloids, phenolics, flavonoids, terpenoids, pigments, and essential oils (Chakraborty and Hancz 2011). Thus, researchers have investigated ways to apply EOs to improve immunity and disease resistance in fish (Bulfon et al. 2015). Blood leukocytes and proteins are essential for the proper functioning of the immune system of fish, and their effects are different from those achieved by administering herbal immunostimulants (Chakraborty and Hancz 2011). In this study, the serum levels of total protein, albumin, and globulin were lower, but the albumin/globulin ratio was higher in the fish cultured under HSD compared to that in the fish cultured under LSD. Adding LEO to the diet increased the total protein, albumin, and globulin serum levels in fish cultured under HSD and LSD. The albumin/globulin ratio was significantly higher in the HSD + LEO0 group. The increase in the blood protein levels may be due to an improvement in the health and function of the fish liver, as this organ is the site of synthesis of many proteins (Hoseini and Tarkhani 2013); lavender essential oil has hepatoprotective effects on the liver (Selmi et al. 2015). An increase in the total protein level after administering LEO suggested that essential oils can enhance protein absorption and digestion (Krishan and Narang 2014), increase the efficacy of protein utilization, and promote weight gain. Additionally, Houghton et al. (1995) showed that globulin levels can increase due to the beneficial effects of aromatic compounds on the immune function, specifically on the promotion of cell growth and defense and the inhibition of non-enzymatic oxidation.

We also found that the serum levels of LYZ, NO, and C3 were lower in the fish cultured under HSD compared to their levels in the fish cultured under LSD. However, adding LEO to the diet significantly increased the serum levels of LYZ, NO, and C3 in fish cultured under LSD and HSD; the effect of 1% LEO was higher than the effect of 0.5% LEO. The effect of LEO as an immunostimulant can be attributed to its bioactive components, which include monoterpenoids and monoterpenes (Šimunović et al. 2020; da Silva Rivas et al. 2012; Zhang et al. 2022). In addition, daidzein has been reported to improve the immunity of stressed fish (Furna 2019). Yousefi et al. (2020) showed that dietary lavender extract can significantly increase soluble immune components (plasma globulin, total

Ig, and lysozyme) and cellular innate immune components (leukocytes). Fazio et al. (2017) reported improved phagocytosis, respiratory burst, and peroxidase activities in the head kidney leukocyte of gilt-head sea bream (*Sparus aurata*) in vitro, following the administration of lavender extract (*Lavandula multifida* and *L. angustifolia*). The improvement in the innate immune system of fish might be due to an improvement in the overall health. Stress can deteriorate the general health and reduce the immune function of fish (Tort 2011). Lysozyme activity may increase due to an increase in the production of neutrophils and lysozymes (Costas et al. 2011).

In this study, we also evaluated the resistance of the Nile tilapia to *A. veronii* infection at the end of the feeding trial. Fish in the LSD + LEO1 group showed the highest survivability rate (86.66%), followed by those in the LSD + LEO0.5 group (83.33%). LEO addition increased the survivability of fish cultured under HSD; specifically, the survivability rates were 76.66% and 73.33% in the HSD + LEO1 and HSD + LEO0.5 groups, respectively, which were higher than that recorded in the HSD + LEO0 group (53.33%). The antimicrobial activity of LEO may be due to the presence of α-pinene, the main bioactive component of LEO (32.24%) (Šimunović et al. 2020; Oliveira and Santos 2021), α-linalool (Jabir et al. 2018), α-terpineol (Badawy et al. 2019), eucalyptol (Di et al. 2022), and 9,12,15- octadecatrienoic acid (Al-Gara et al. 2019).

Cytokines in animals are markers of inflammation caused by stressful conditions, such as high stocking and transportation densities (Yarahmadi et al. 2016; Hoseini et al. 2019). We found an increase in the level of expression of immune-related genes (*IL-1β*, *IL-6*, *IL-8*, *NF-k*, and *TLR-4*) in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups. The expression of *TGF-β* and *IL-10* was upregulated in fish that were administered 1% and 0.5% LEO and cultured under LSD and HSD. These results suggested that LEO has anti-inflammatory effects, through which it improves the health of fish. The anti-inflammatory properties of LEO (Silva et al. 2015; Cardia et al. 2018) are probably imparted by components such as linalool and cineole. Linalool acts by saturating the receptors involved in the inflammatory reaction, for example, *N*-methyl-D-aspartate (NMDA) receptors (Peana et al. 2002), whereas cineole reduces corticosteroid production and prevents immunosuppression (Juergens 2014). Corticosteroid production increases under stress and significantly inhibits the production of many cytokines and antibodies, thus reducing the functions of the innate immune system (Tort 2011). We also found that the immunoexpression of *NF-kB* and *IL-1β* increased significantly in tissues after 1% LEO was administered to fish cultured under HSD and LSD. These results indicated that LEO ameliorated the immune status impaired by HSD and strengthened the immunity of fish cultured under LSD.

Stress factors, such as overcrowding, may increase oxidative stress related to ROS accumulation, resulting in lower SOD and CAT activities. These enzymes are the first line of defense against oxidative stress and degrade oxidizing molecules (superoxide and hydrogen peroxide). The decrease in the activity of these enzymes leads to lipid peroxidation and the formation of MDA (Yousefi et al. 2018a). Thus, the presence of ROS in cells leads to the activation of biochemical reactions that decrease cellular functions because of oxidative damage to lipids, proteins, and carbohydrates. These changes can cause apoptosis and accumulate oxidized molecular groups. The oxidative stress framework is stimulated by an imbalance in the production of oxidants and antioxidants, as determined by low levels of antioxidant enzymes and high prooxidant levels; these changes decrease the response of the immune system (Biller and Takahashi 2018). In this study, fish cultured under HSD showed lower antioxidant activity, indicated by a reduction in CAT, SOD, and GST levels and an increase in the MDA levels, compared to the fish cultured under LSD. However, adding LEO to the diet improved the antioxidant

activity (CAT, SOD, and GST) in the LSD + LEO1 and LSD + LEO0.5 groups, followed by the HSD + LEO1 and HSD + LEO0.5 groups compared to the activity recorded in the LSD + LEO0 and HSD + LEO0 groups. We also found that the expression of apoptosis-related genes (*caspase 3* and *BAX*) was upregulated in the HSD + LEO0 group and was significantly altered by the addition of 1% LEO. In contrast, *BCL-2* expression was downregulated in the HSD + LEO0 group and significantly upregulated after 1% LEO was added. The level of expression of *BAX/BCL-2* was the highest in the HSD + LEO0 group and the lowest in the LSD + LEO1, LSD + LEO0.5, and HSD + LEO1 groups. The antioxidant activity of LEO may be due to the presence of monoterpenes, such as α-linalool, acetic acid linalool ester, and α-pinene, and monoterpenoids, such as eucalyptol, phellandral, and terpineol, which are known to have antioxidant and antimicrobial activities (Carrasco et al. 2016; Bose et al. 2020; Beier et al. 2014; Puvača et al. 2019). Isoflavones such as daidzein have the ability to control several physiological processes such as lipid metabolism, inflammation, apoptosis, and the hypothalamus-pituitary axis by acting through estrogen receptors (Pilšáková et al. 2010). Moreover, the flavonoid compounds in LEO such as quercetin have been reported for their antioxidant, anti-inflammatory, and hepatoprotective effects (Mansour et al. 2022).

Oxidative stress was also reported in another study on overstressed rainbow trout, *O. mykiss* (Mirghaed et al. 2018). Yousefi et al. (2020) reported that lavender extract has strong antioxidant properties, indicated by higher antioxidant enzyme activities and lower MDA levels in the fish. Several in vitro studies have found such antioxidant effects (Gülçin et al. 2004; Kıvrak 2018), which might be due to the content of the antioxidant compounds linalool (Yousefi et al. 2018b; Amer et al. 2022a) and cineole (Mirghaed et al. 2018; Ciftci et al. 2011). Yousefi et al. (2020) found that 1.5% lavender extract can prevent oxidative stress produced by over-crowding stress, as it can inhibit significant changes in the SOD activity and MDA content under crowding stress. The results were similar to those reported by Mirghaed et al. (2018), who found that dietary intake of cineole significantly prevented oxidative stress after overcrowding in the rainbow trout, *Oncorhynchus mykiss*.

Conclusion

To summarize, our findings indicated that adding LEO to the diet of the Nile tilapia acts as an anti-stress factor under high stocking densities. Dietary LEO can increase the growth of fish under stress by enhancing the activity of their digestive enzymes, increasing circulating growth hormone levels, improving health, and preventing tissue damage. When LEO was administered to fish under stress, it boosted the immune status by strengthening the innate immune system and increasing pro-inflammatory and anti-inflammatory cytokines. LEO addition improved the health of fish by reducing oxidative stress and apoptosis. It also increased the resistance of fish to *A. veronii* infection. We found that the level of LEO that should be added to the diet of fish cultured under HSD is 1%, considering that this concentration showed the best results for mitigating the negative effects of HSD.

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Author contribution SAAA and ME designed the study. Methodology: SAAA and ME; SAA, TK, EEN, EMY, A-WAA-W, and SJD. Software and data curation: SAA and SAAA. Writing—original draft

preparation: SAA and SAAA. Writing—reviewing and editing: SAA and SAAA. All the authors approved the final draft.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethical approval The Institutional Animal Care and Use Committee of Zagazig University in Egypt accepted the experimental protocol (ZU-IACUC/2/F/299/2023).

Competing interests The authors declare no competing interests.

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